
بسم الله الرحمن الرحیم
IN THE NAME OF GOD
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«12th Journal Club»

Ultrasound-triggered herceptin liposomes for breast cancer therapy

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➢ About the Journal

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Subject	Rank	Quartile	Percentile
Multidisciplinary Sciences (SCIE)	17/73	Q1	N/A

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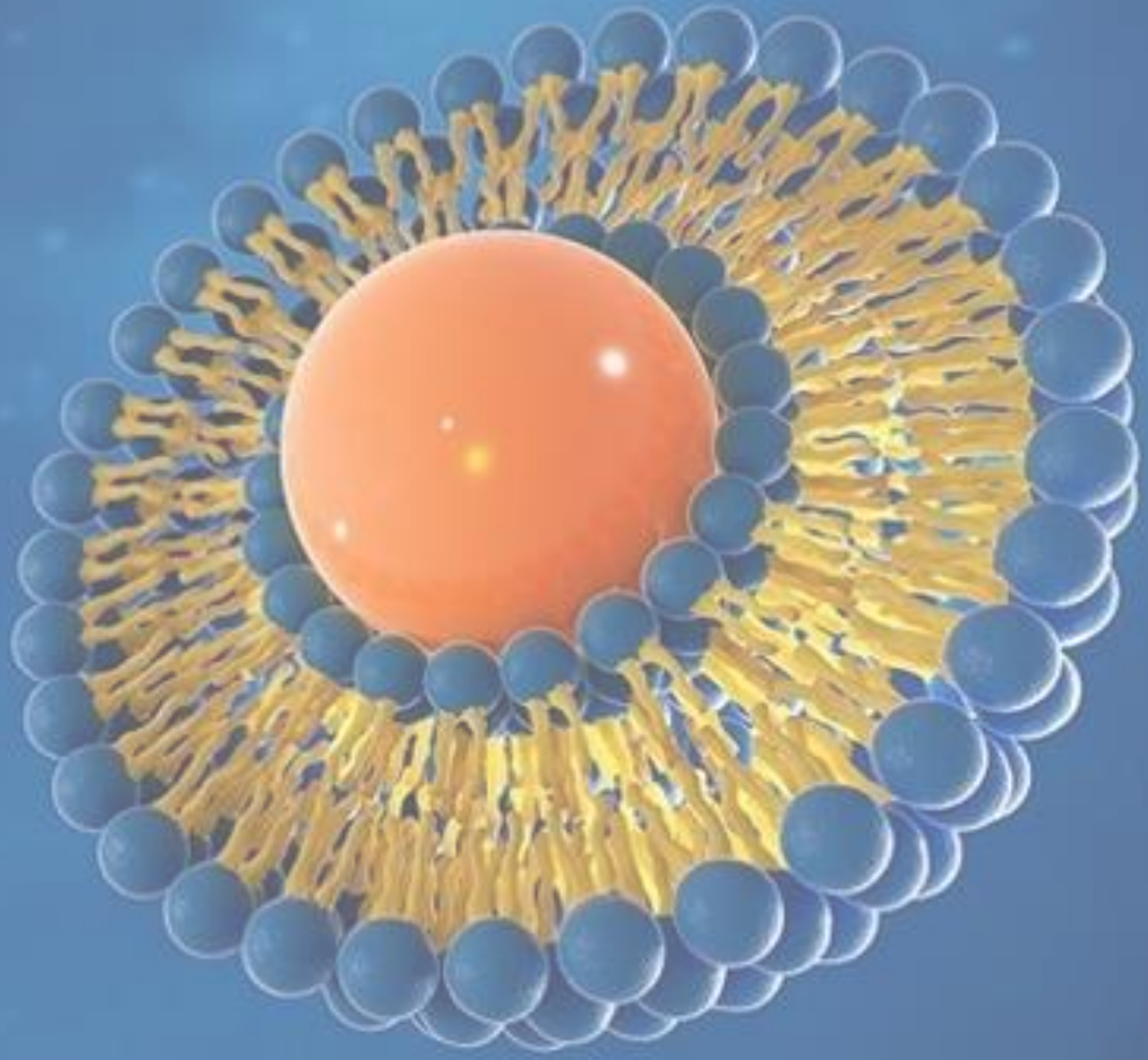
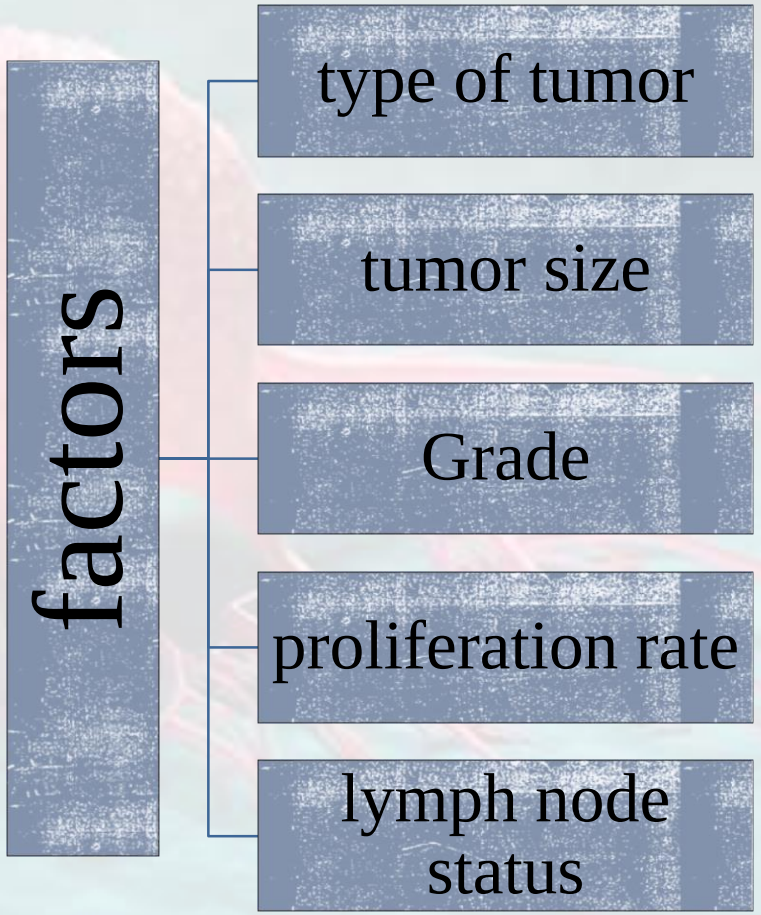
Subject	Rank	Quartile	Percentile
Multidisciplinary	8/110	Q1	93%

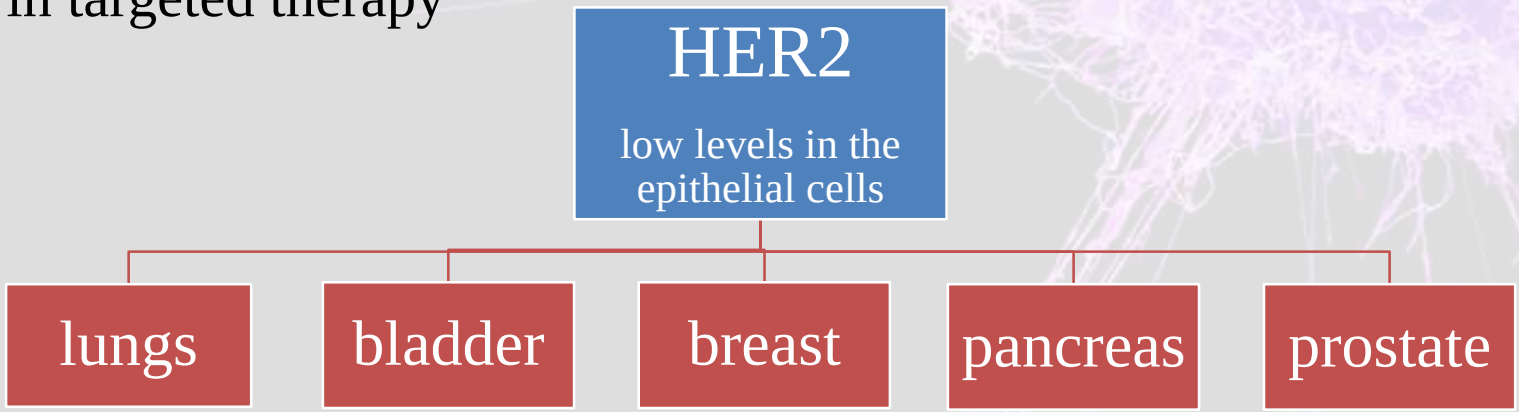
Introduction



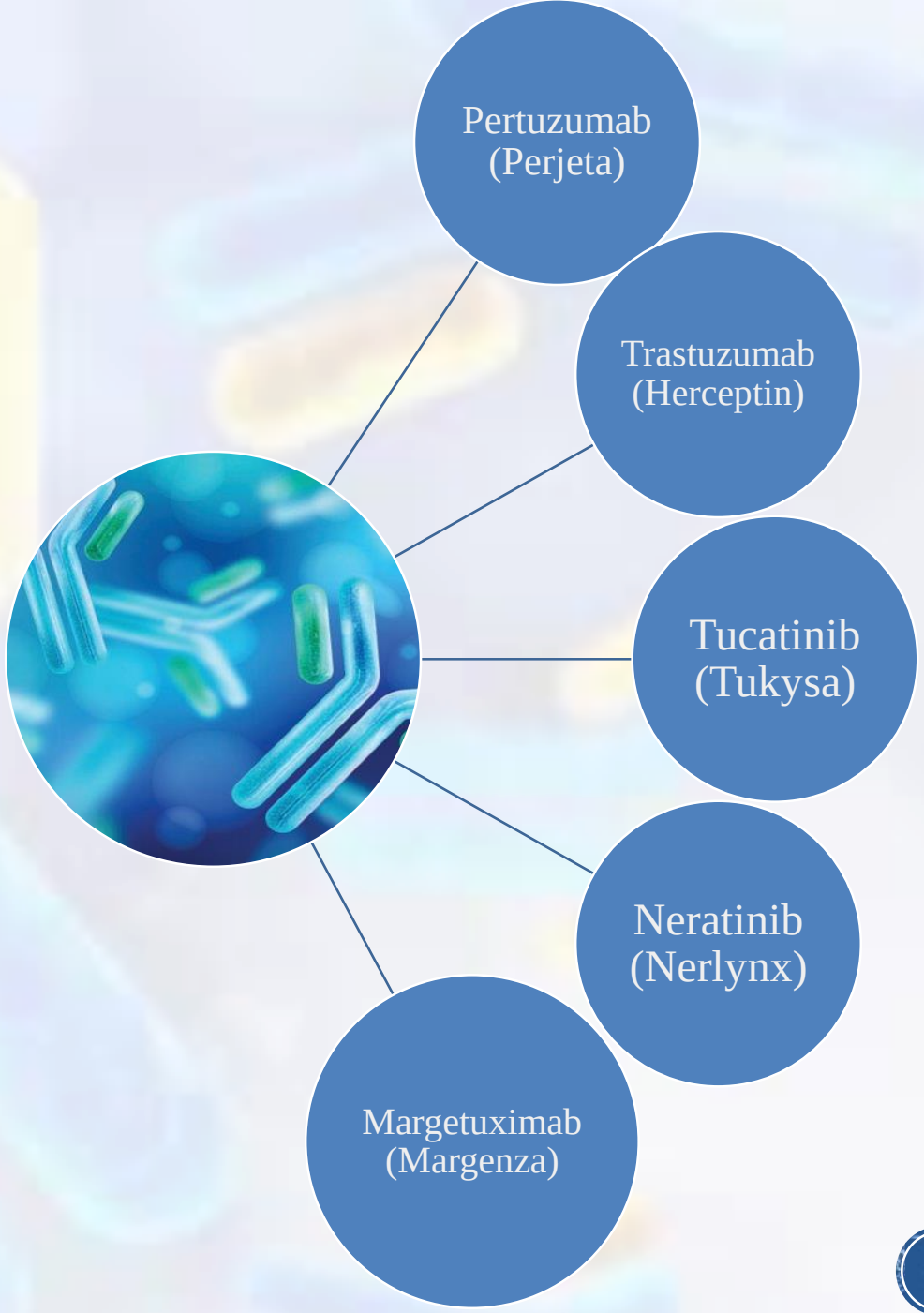
- Breast cancer is the most common type of cancer in females ➡
- treatment is complex and relies on several factors
- For early-stage breast cancer ➡ usually surgery
- For more aggressive tumors ➡ chemotherapy or hormonal therapy before surgery
- After surgery ➡ to lower the risk of recurrence ➡ adjuvant therapies include radiation therapy, chemotherapy, hormonal therapy, immunotherapy and/or targeted therapy



- In recent years, the presence of breast cancer tumor markers has been investigated
- estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2)
- HER2 overexpression ⇒ malignant progression and cancer
- HER2 overexpression ⇒ in approximately 25% of all breast cancers ⇒ more aggressive disease and endocrine therapy resistance
- an appealing in targeted therapy



- One of the significant advancements in breast cancer treatment ➡ mAb-based therapies
- The humanized monoclonal antibody Trastuzumab (TRA), sold under the brand name Herceptin among others, was FDA approved in 1998 for the treatment of HER2-positive breast cancers
- TRA ➡ enhance the effects of chemotherapy reduce the risk of recurrence when used as an adjuvant therapy



- TRA ⟹ a humanized IgG(1) kappa monoclonal antibody
- 145.5 kDa
- high and specific affinity towards HER2 receptors
- stop tumor development cell signaling
- reduced PI3K/Akt signaling
- enhanced degradation of HER2 receptors
- antibody-dependent cellular cytotoxicity (ADCC)

- TRA → is currently used as a first-line treatment of breast cancer
- However, drug resistance is inevitable
- alternative treatment options → Anti-HER2 immunoliposomes combine the tumor-targeting properties of mAbs, with the drug delivery properties
- offering prolonged inhibition of the HER2 pathway
- reducing drug side effects
- increasing blood circulation time
- increasing drug concentrations at target sites

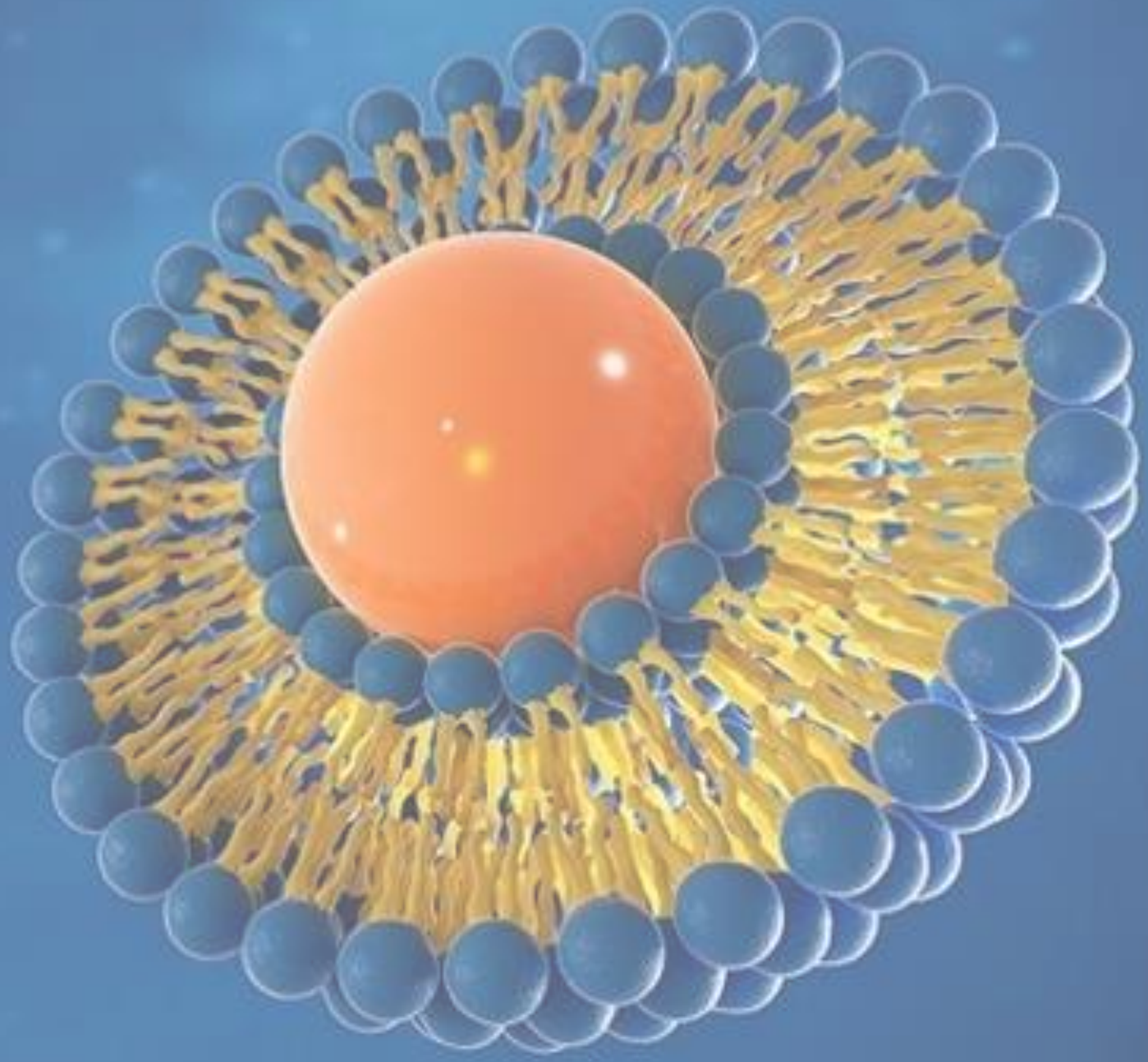
- Liposomes are one of the most widely used nanocarriers in drug delivery
- nanosized to microsized
- spheres of phospholipid bilayers separated by aqueous compartments
- the circulation-time of liposomes can be increased and their detection by the reticuloendothelial system (RES) reduced by conjugating stealth-imparting polymers to their surfaces
- The most widely used polymeric substance ⟹ poly-ethylene glycol (PEG)

- Antibody therapeutics have revolutionized the treatment of cancer over the past few decades
- Antibodies in targeted therapy ➡ to deliver potent chemotherapeutic agents in the form of antibody–drug conjugates (ADCs) or as targeting ligands decorating the surfaces of nanocarriers
- Immunoliposomes provide a complementary and more advantageous, drug delivery strategy to ADCs
- The large size of ADCs often hinders their diffusion into the intratumoral space
- immunoliposomes ➡ higher drug-carrying capacity (20,000–150,000 drug molecules/liposome) compared to ADCs

- SDDSs in their target sites ⟹ release their contents when exposed to either an internal or external stimulus
- the trigger in this work ⟹ ultrasound (US)
- US ⟹ a cyclic sinusoidal acoustic wave
- frequencies higher than those of the human hearing range (> 20 kHz)
- the biological effects of US can be either :
- thermal ⟹ are caused by energy dissipation
- Mechanical ⟹ occur because of the acoustic wave propagation and pressure variations
- The mechanical effects of US form gas bubbles due to changes in pressure

- In this study:
- calcein and Doxorubicin (DOX)
- loaded pegylated liposomes
- with TRA
- to target breast cancer cells
- LFUS applied to trigger the release of calcein and DOX from the liposomes
- enhancing their uptake by the cancer cells

Methods & Results



Preparation of control liposomes

- using the thin-film hydration method

cholesterol, DPPC, and DSPE-PEG(2000)-NH2 at molar ratios of 30:65:5
4 ml chloroform

evaporated 50 ℃ for 15 min

hydrated 2 ml of a 30-mM calcein solution pH=7.4

To obtain unilamellar vesicles sonicated 2 min 40-kHz sonicator bath

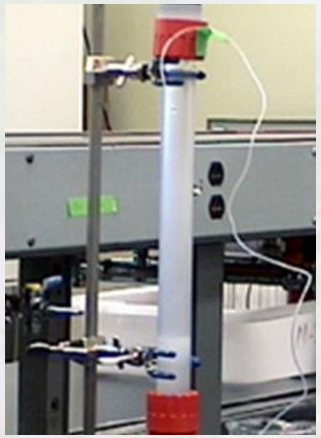
extruded 200-nm polycarbonate filters

purification Sephadex G-100 column

collected fractions were stored at 4 °C

- same protocol liposomes encapsulating ammonium sulfate
- The ammonium sulfate method was used to load DOX into the formed liposomes

0.11-M solution of ammonium sulfate pH=5.5 to hydrate the dry lipid film



Liposomes were purified with a Sephadex G-25 gel filtration column (previously equilibrated with HEPES buffer)

DOX was added (DOX to lipid ratio of 1:6 (w/w))

keeping in a water bath
60°C
45 min
mild stirring

centrifuging through a Sephadex G-25 gel filtration column

Preparation of Trastuzumab (TRA)-liposomes

Calcein and DOX liposomes were prepared → cyanuric chloride was dissolved in acetone 10 mg/ml solution → 9.23 µl was diluted in 0.5 ml deionized water →

was added to 1 ml of the liposomal formulations → pH=8.5 0°C → stir for 3 h → substitution of the chloride particle on the cyanuric chloride with the proton of the NH2 group of the liposomes

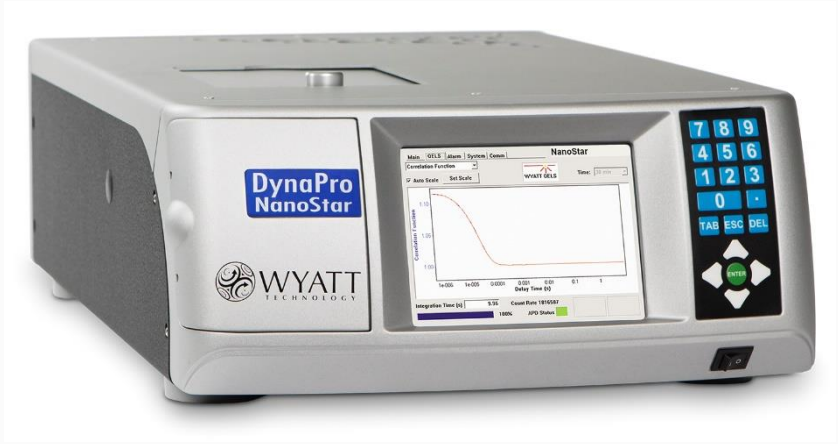
- the linking of the N-terminus on the amino acids of TRA to cyanuric chloride

→ 1 mg TRA in 0.5 ml borate buffer → pH 8.5 → was added to the liposomes → stirring overnight → excess TRA and any free drugs were purified in a Sephacryl S-200 h column

→ stored at 4 °C

Particle size and polydispersity evaluation

- particle size
- polydispersity index(PDI)
- 25 °C
- using DynaPro NanoStar



DynaPro NanoStar Dynamic Light Scattering

➢ Result 1

Liposomes characterization

Liposomes	Radius (nm)		pd%	
	Calcein	DOX	Calcein	DOX
Control liposomes	89.54 ± 0.50	91.2 ± 1.47	11.28 ± 1.11	12.3 ± 3.01
TRA-liposomes	101.10 ± 1.13	94.9 ± 1.29	17.22 ± 2.34	11.2 ± 0.55

Table 1. Size and polydispersity of Calcein and DOX-loaded control and immunoliposomes.

Estimation of phospholipid content

- using the Stewart Assay

liposome samples were dried under vacuum → dissolved in chloroform → was sonicated to break the liposomes

centrifugation step resulted in a biphasic system →

solution was added to 2 ml of ammonium ferrothiocyanate →

the top dark layer was discarded the bottom clear chloroform layer its optical density measured →

using UV–Vis spectroscopy at A_{max} = 485 nm against chloroform as a blank

Trastuzumab conjugation to the liposomes

- Bicinchoninic Acid Assay (BCA)

BCA reagent was prepared : mixing QuantiPro QA buffer, QuantiPro QB, and CuSO4 in a ratio of 25:25:1

1mL of the reagent was added to 1 ml of PBS and 100 µl of the liposomal solution incubation

60°C

1 h

The optical density 562 nm

➢ Result 2

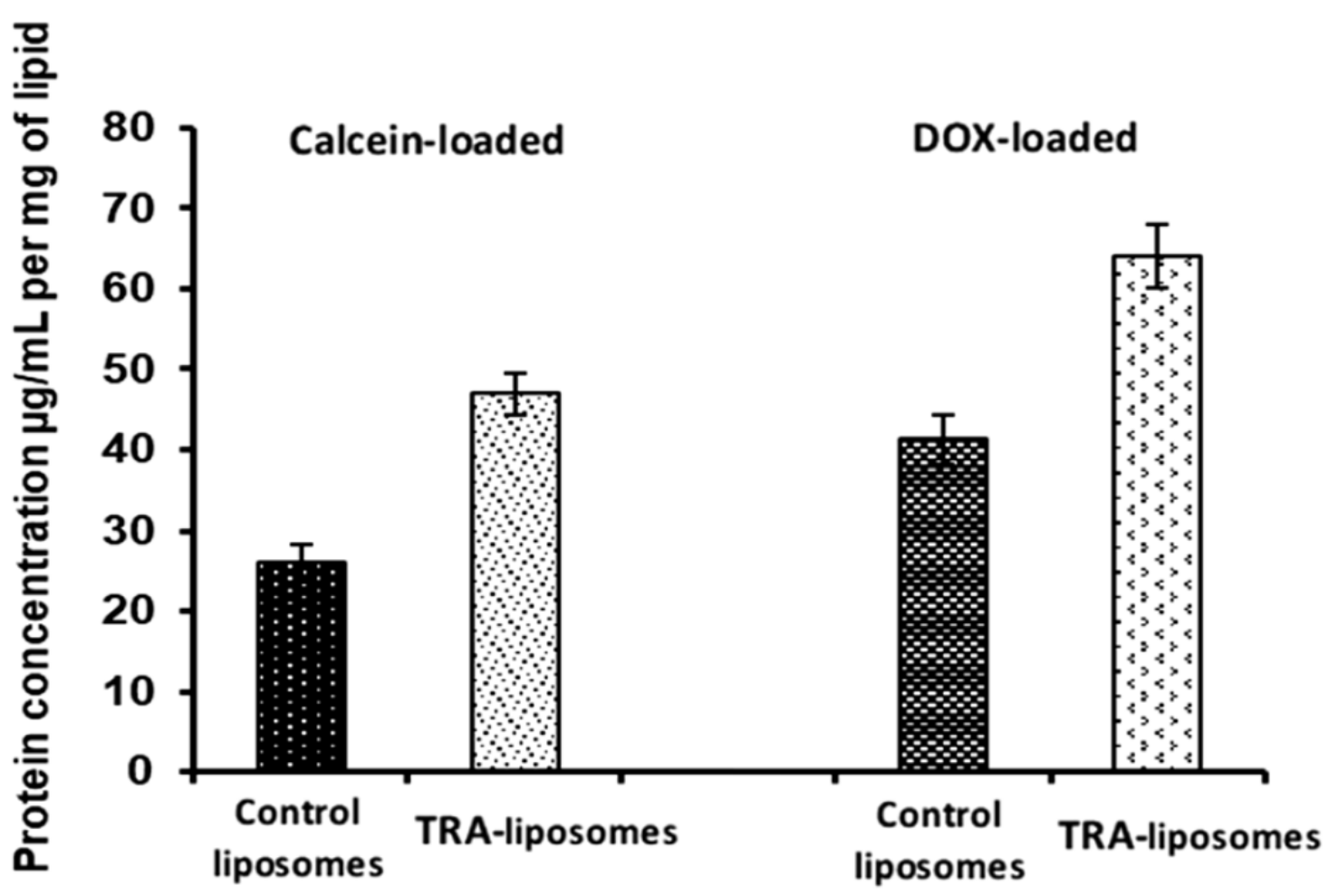


Figure 1. Protein concentrations (per mg of lipids) for the control and TRA-liposomes in both calcein and DOX-loaded liposome.

Power density measurements

ultrasonic probe produced LFUS (at 20 kHz)

power amplitude: 20%, 25% and 30%

Measuring the power densities: hydrophone

- the waves produced by the US create a pressure variation
- be detected by hydrophone and converted into voltage signals

The signals are fed to a digital storage oscilloscope

analyzed MATLAB software

A 2D map was created by keeping the hydrophone at a constant depth

- The measured voltage signals were converted into acoustic pressure in Pascal using the equation:

$$P = \frac{Vrms\,(\boldsymbol{V})}{Voltage\ Sensitivity\left(\frac{\mu V}{Pa}\right)}$$

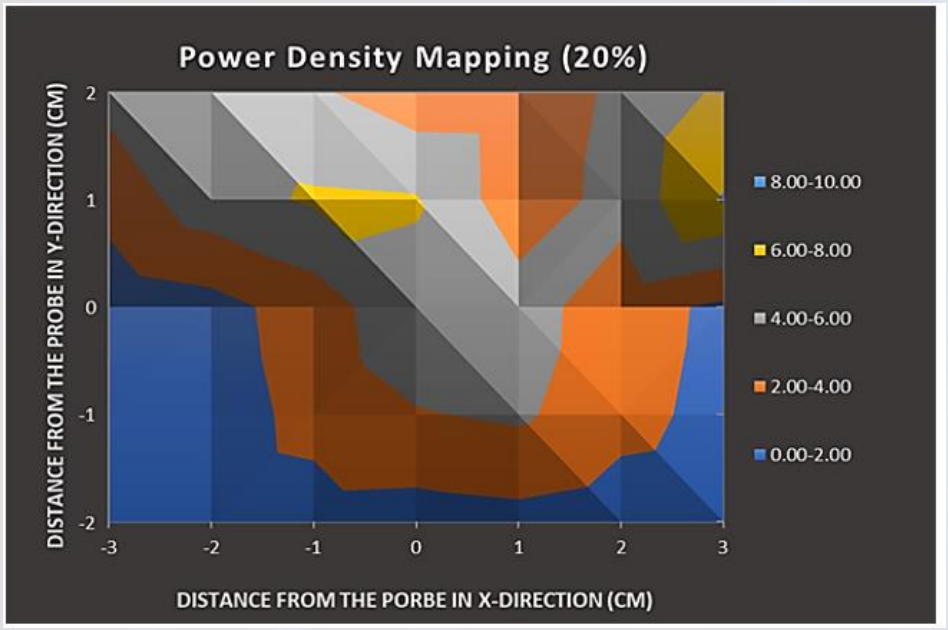
- The value of the hydrophone voltage sensitivity was provided/reported by the hydrophone manufacturer as 30 μV/Pa
- the ultrasound power density 'I', in Watt/cm^2, is given by the equation:

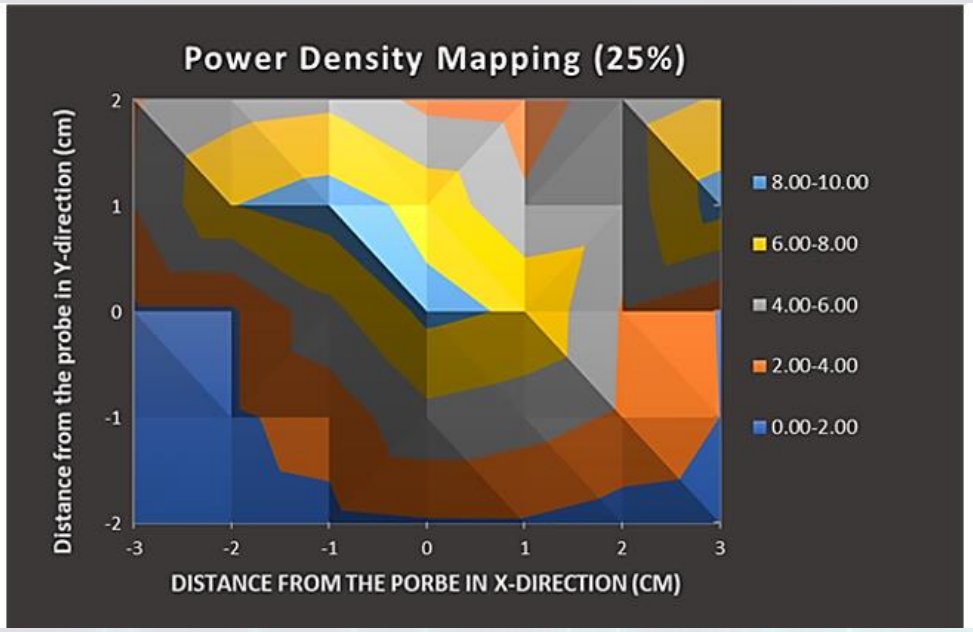
$$PowerDensity, I = \frac{P^2}{Z}$$

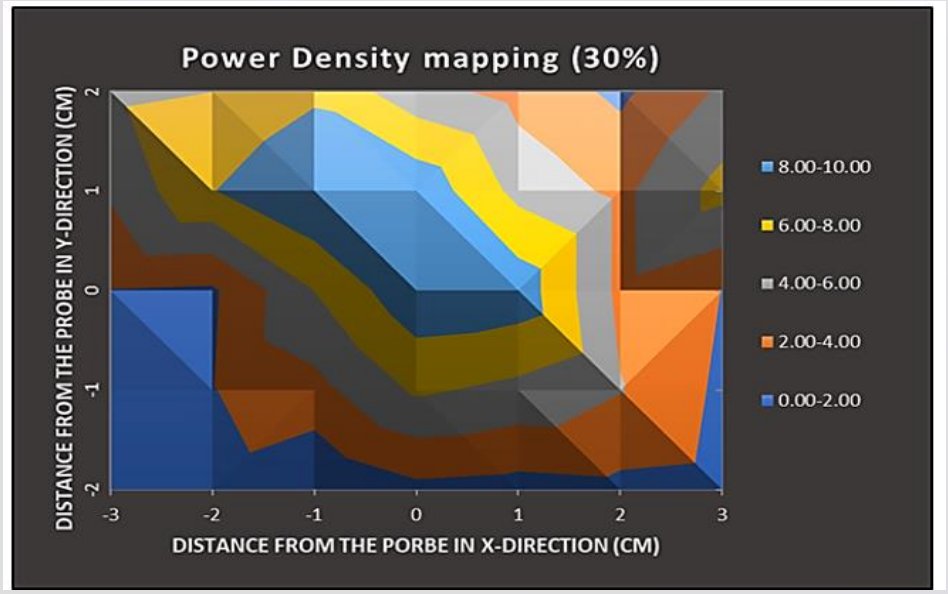
- 'Z' is the acoustic impedance of the medium (the impedance of water= 1.48×10^6)
- 'P' is the pressure measured in Pascals

➢ Result 3

Determination of the different power densities of the LFUS







- Power densities values measured with the hydrophone at various distances from the probe at 30%, 25% and 20% amplitude.

Low-Frequency ultrasound release studies

- The release of calcein and DOX from liposomes was triggered 20-kHz low-frequency ultrasonic probe

monitored by fluorescence changes using a Spectrofluorometer

	excitation	emission
Calcein	495 nm	515 nm
DOX	~485 nm	595 nm

75 µL of liposomes in 3 mL of PBS in a fluorescence cuvette

The initial fluorescence intensity I_0 was measured for 60 s before sonication

US with 20 s on and 10 s off for calcein liposomes 20 s on, 20 s off for DOX-liposomes

to account for the thermal effects of US three different power densities, 6.2, 9 and 10 mW/cm^2

The pulsed US cycles were continued until a fluorescence plateau

50 µL of Triton X-100 were added to lyse the liposomes (simulating 100% drug release)

- The percent of drug released from the liposomes ➡ Cumulative Fraction Release (CFR)

$$CFR = \frac{I_t - I_o}{I_\infty - I_o}$$

- I_0 the baseline intensity
- I_t the intensity at time
- $I\infty$ the highest fluorescence intensity value obtained

➢ Result 4

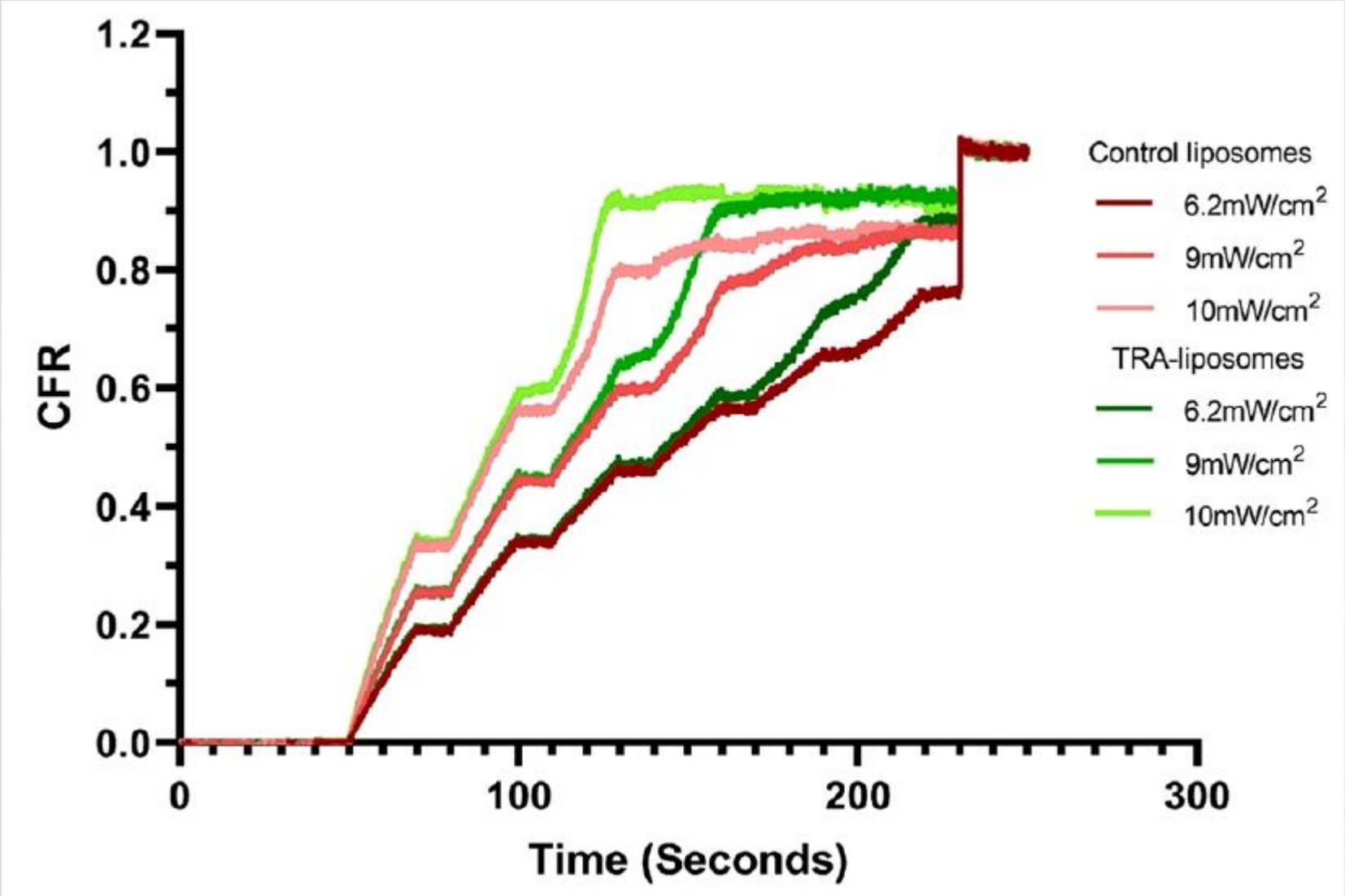


Figure 2. Normalized release profiles of the control and TRA-liposomes loaded with calcein at 6.2 mW/cm^2, 9 mW/cm^2 and 10 mW/cm^2

➢ Result 4 Low-frequency ultrasound (LFUS) triggered release

- TRA-liposomes were more acoustically sensitive ⟹ releasing around 92%
- the control liposomes ⟹ 86% released
- the presence of the TRA molecules ⟹ slightly destabilize the membrane

  making the liposomes more susceptible to acoustic mechanical waves

➢ Result 5

$$P_{neg} = \sqrt{2IZ}$$

- the mechanical index (MI) ⇒ parameter to indicate the possibility of the occurrence of cavitation
- The negative pressure is dependent:
- the acoustic impedance of water, Z, (1.48 MPa sec/m)
- the intensity of the LFUS, I,

LFUS power densities (mW/cm^2)	MI values
6.2	0.096
9	0.115
10	0.121

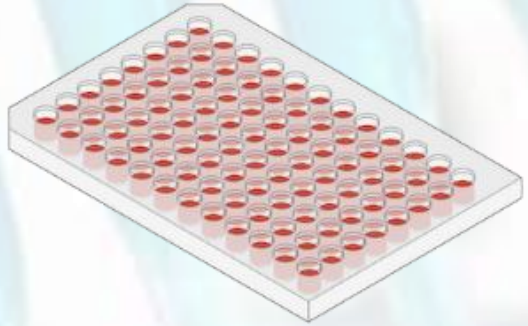
- The threshold of

collapse cavitation	**MI = 0.3**
biological effects	**MI > 0.7**
tissue damage	**MI >1**

MTT assay

SKBR3 (HER2 + cells) → DMEM
MDA-MB-231 (HER2- cells) → RPMI

- 10% FBS
- 1% penstrep
- 37 °C
- 5% CO2



1×10^4 cells per well
96-well plates

24 h

different treatments:8 μM per well (n=3)

- free DOX
- DOX-loaded control liposomes
- DOX-TRA liposomes
- Drug-free control liposomes as neg control

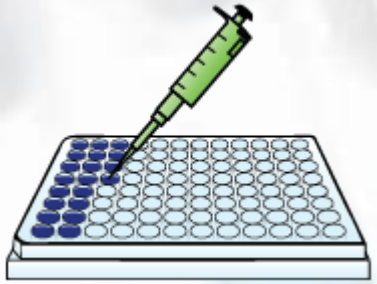
were incubated 4 h

LFUS for 5 min in a 35-kHz bath

One plate of each cell line was not subjected to LFUS to serve as a control

incubation

48 h



medium was replaced with the MTT medium

37 °C

4 h

100 µl of DMSO

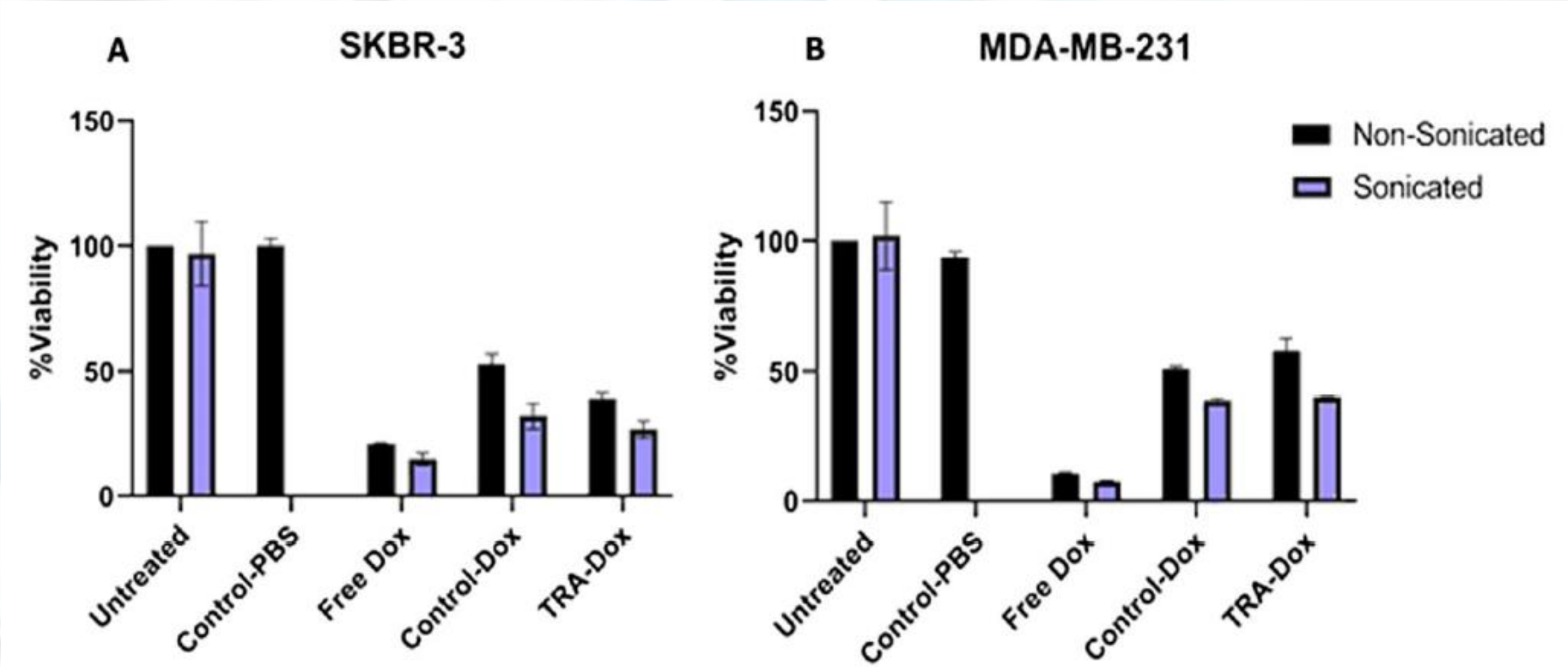
15 min

absorbance values
microplate reader at 570 nm

➢ Result 6 In vitro cytotoxicity analysis

- liposomes without DOX encapsulation were used as control → cell viability (~ 100%)
- cytotoxicity is due to the action of DOX and not the liposomes themselves
- the toxicity of LFUS (35 kHz) on the cells was also examined
- no significant effect on cell viability compared to the non-sonicated cells

➢ Result 6


A
SKBR-3
%Viability
150
100
50
0
Untreated
Control-PBS
Free Dox
Control-Dox
TRA-Dox
B
MDA-MB-231
%Viability
150
100
50
0
Untreated
Control-PBS
Free Dox
Control-Dox
TRA-Dox
Non-Sonicated
Sonicated

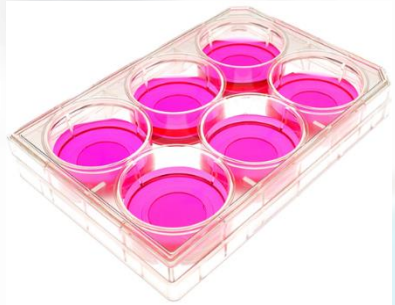
➢ Result 6

%viability

	SKBR3	MDA-MB-231
TRA-liposomes	38.9 ± 2.54%	58.0 ± 1.91%
control liposomes	53.1 ± 3.89%	51.1 ± 0.933%
LFUS+TRA-liposomes[1]	27.4 ± 0.260%	40.1 ± 0.216%
LFUS+control liposomes[1]	32.9 ± 1.31%	38.7 ± 0.858%
DOX+LFUS[2]	14.8 ± 2.52%	Not reported
free DOX (8 μM)	20.8 ± 0.5%	Not reported

1. synergistic effects of using LFUS to trigger drug release
2. sonoporation effect → enhancing the cytotoxic performance of the free drug

Flow cytometry analysis

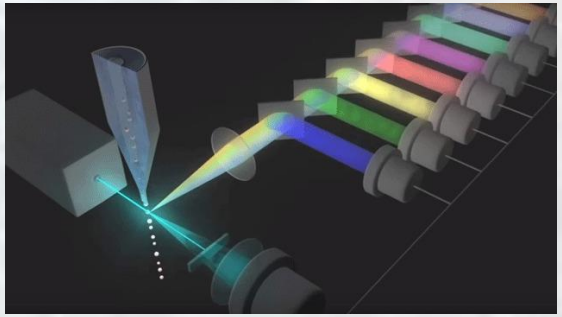


SKBR3 and MDA-MB-231 cells
(2×10^5 cells/ml) in 6-well plates

24 h → treat with the control and TRA-DOX liposomes → 4 h

- LFUS bath 35-kHz 5 min
- power density 20 mW/cm^2
- One plate from each cell line was treated with both types of liposomes without LFUS as a reference

1 h → Trypsin was added to detach the cells → resuspended in PBS →



the flow cytometer analysis

➢ Result 7

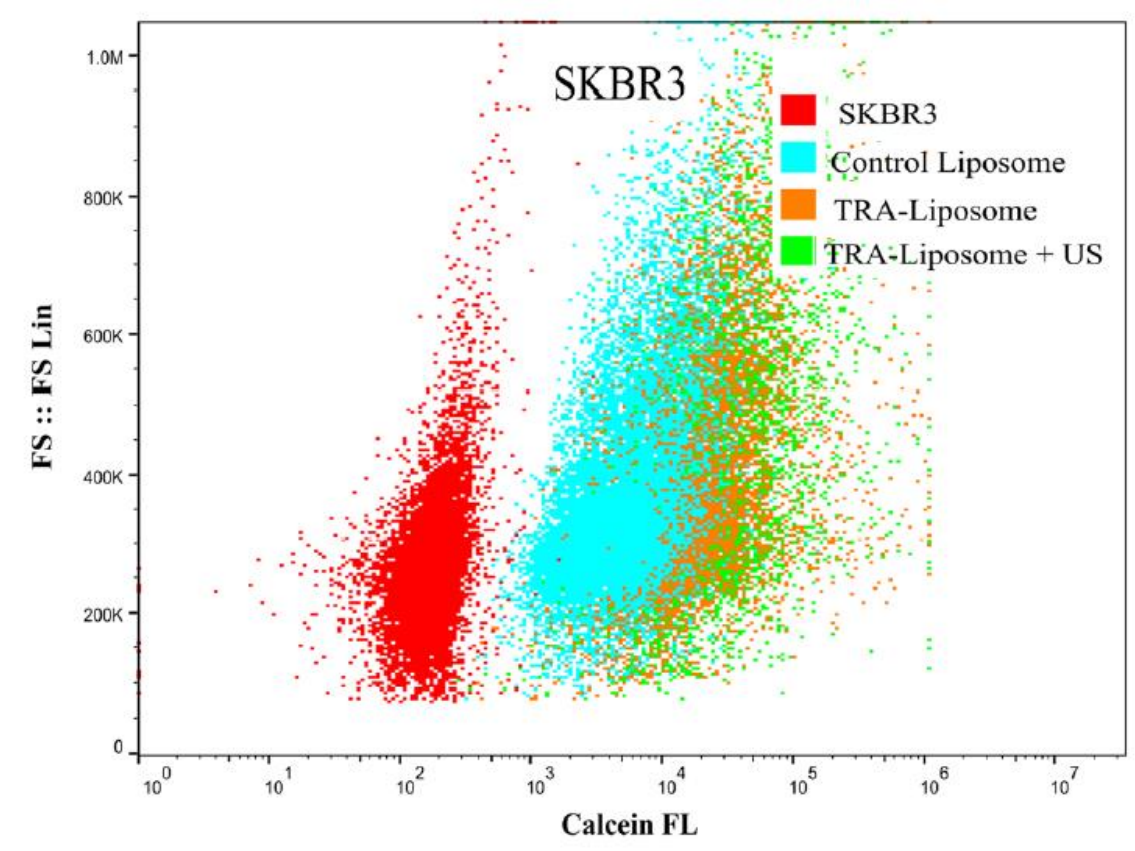
Flow cytometry analysis of cellular uptake of calcein

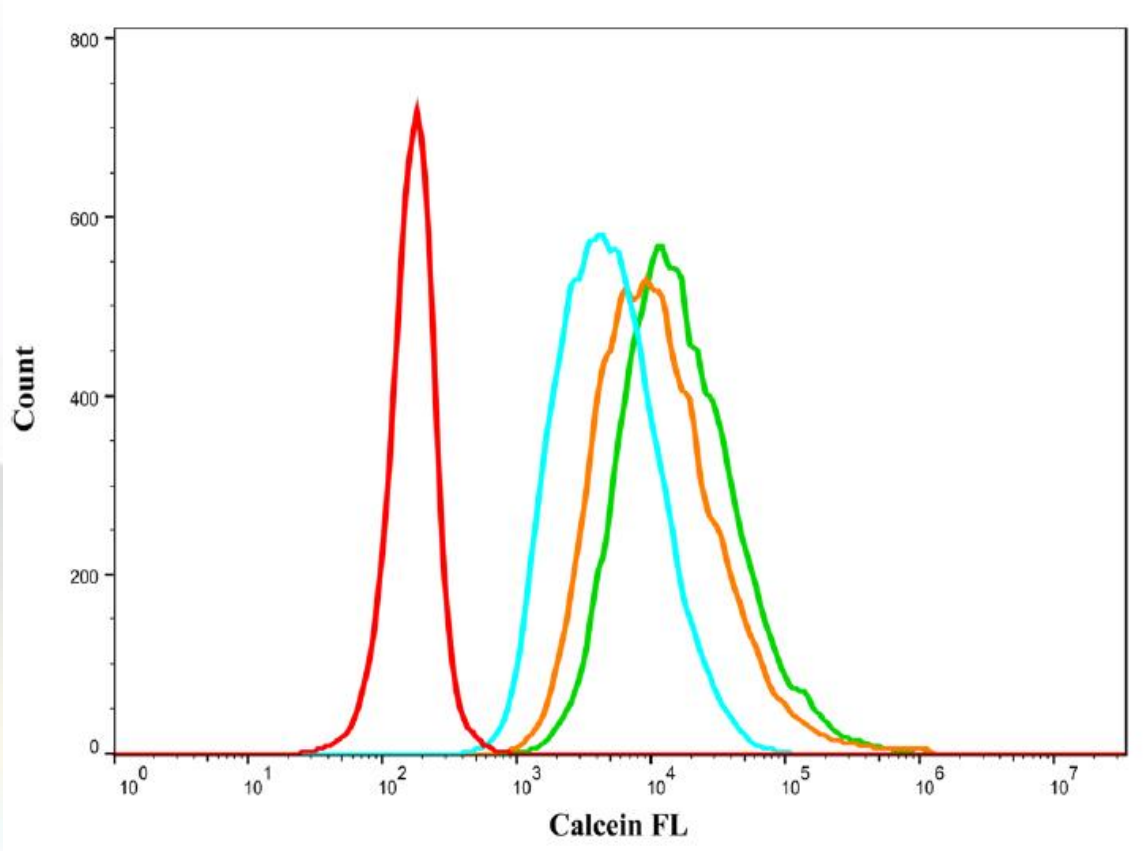
- HER2 + (SKBR3) and HER2- (MDA-MB-231) cells
- incubated with either the control or TRA-liposomes for 4 h
- the average calcein fluorescence intensity in SKBR3 cells
- TRA-liposomes (25,160)
- control liposomes (7236)
- Sonicating the cells with LFUS (35-kHz) for 5 min
- further increase in calcein fluorescent intensity with TRA-liposomes (32,735)
- TRA-liposomes+US compared to control liposomes ⇒ 4.5-fold increase in fluorescence intensity

➢ Result 7

- The recorded increase in the fluorescence intensity of calcein inside SKBR3 cells when incubated with TRAliposomes compared to control liposomes due to the overexpressed HER2 receptors
- same experiment a triple-negative cell line (MDA-MB-231)
- no significant difference inside MDA-MB-231 cells with the control(10,022) or TRA-liposomes(13,914)
- low expression of HER2 receptors on the surface of MDA-MB-231 cells ⇒ reducing the targeting ability of the TRA-liposomes ⇒ low cellular uptake of the calcein

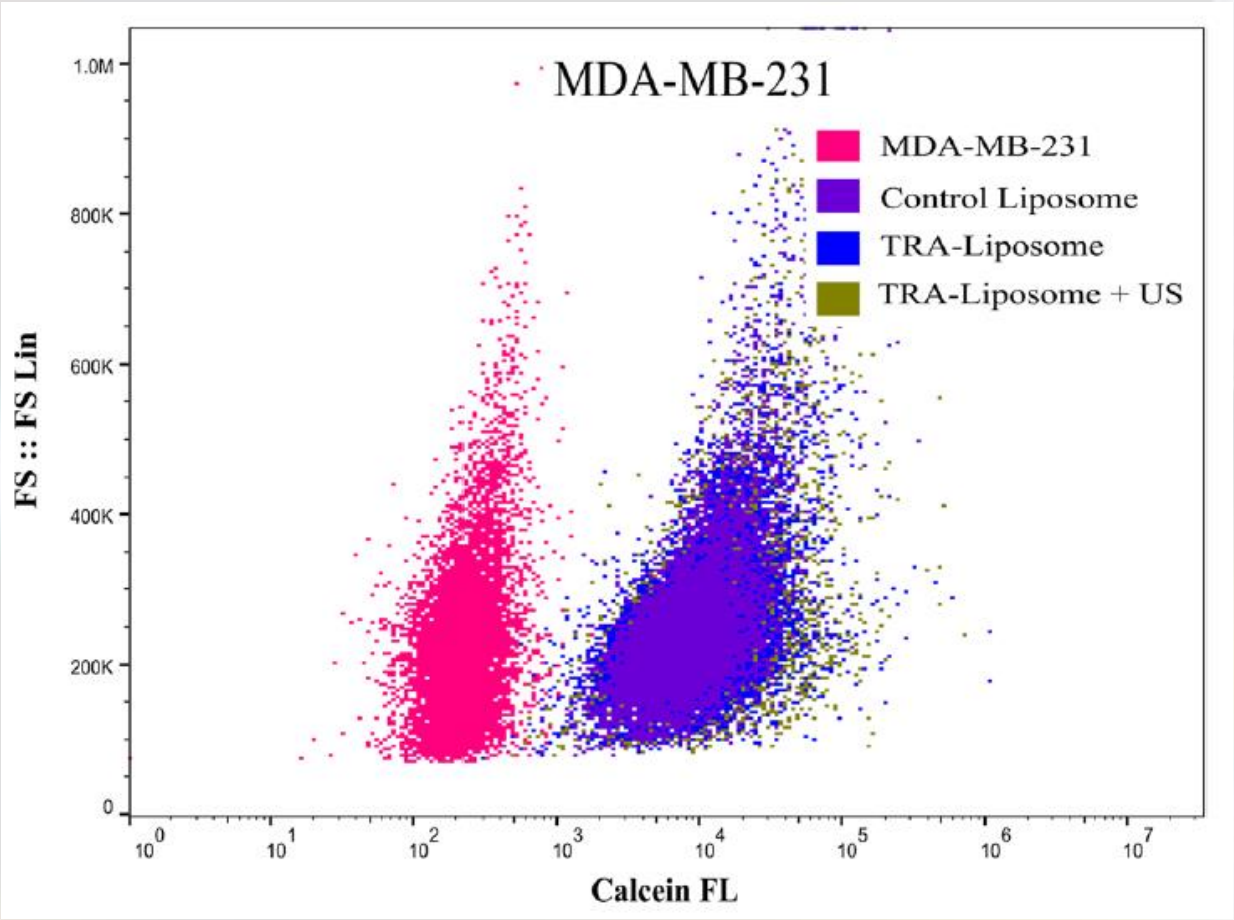
➢ Result 7

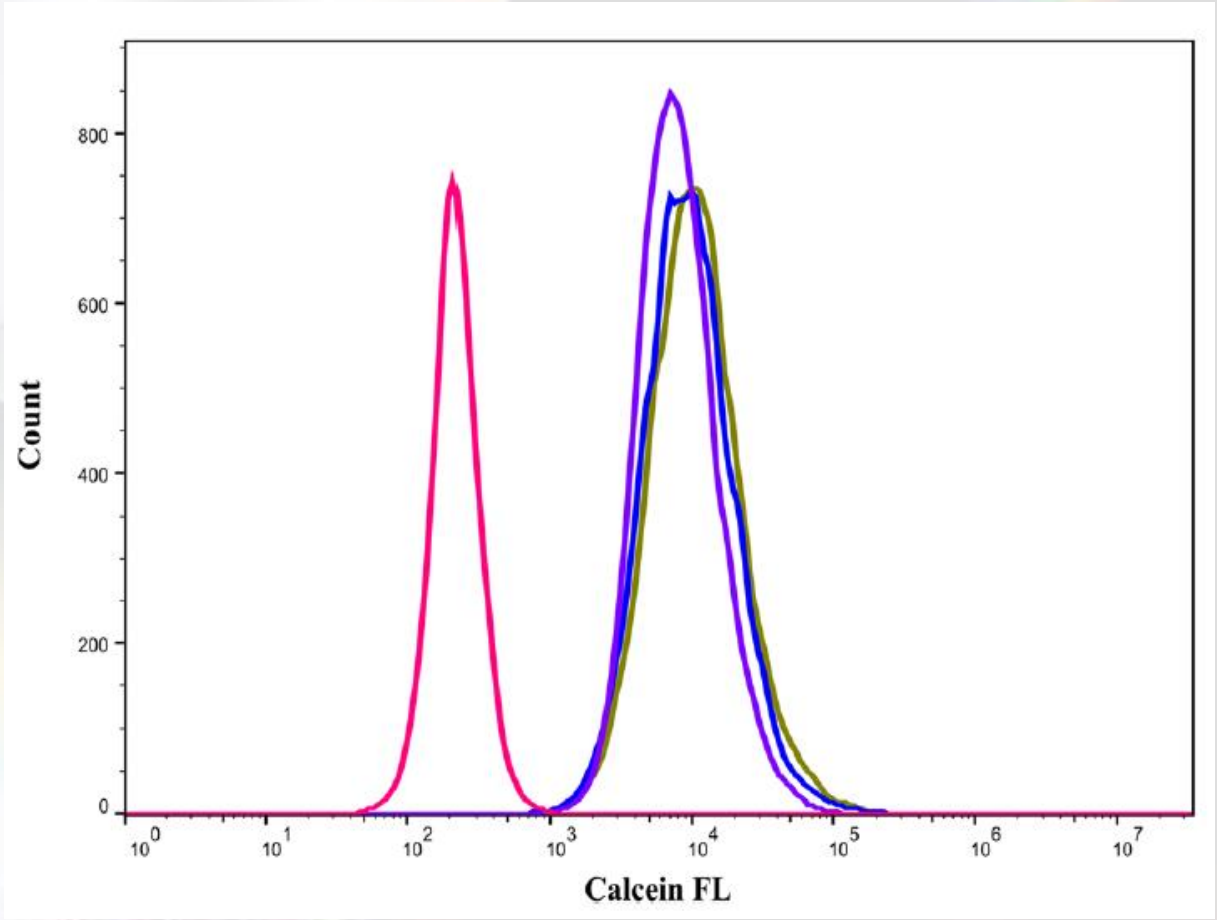




- Flow cytometry analysis of calcein uptake following their incubation with control liposomes or TRA-liposomes followed by ultrasound (US) sonication (35-kHz) for 5 min at a power density of 20 mW/cm^2
- Untreated cells served as a negative control for background fluorescence

➢ Result 7

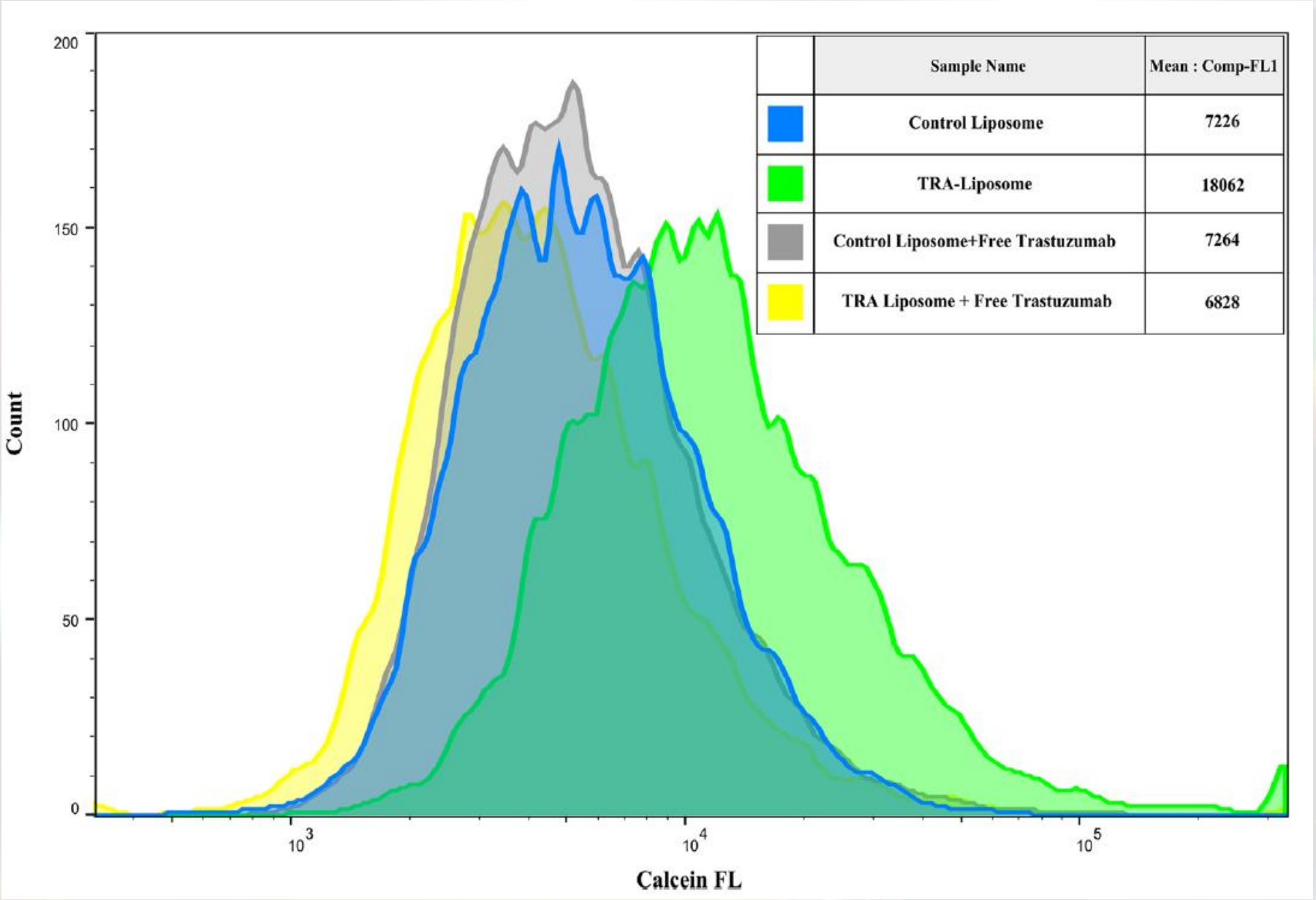

MDA-MB-231
MDA-MB-231
Control Liposome
TRA-Liposome
TRA-Liposome + US
FS :: FS Lin
Calcein FL
1.0M
800K
600K
400K
200K
0


Count
Calcein FL
800
600
400
200
0

➢ Result 8

- The suggested mechanism of the binding of immunoliposomes to HER2 receptors overexpressed on the surface of SKB3 cells was examined
- blocking HER2 receptors to prevent the targeted liposomes from binding
- SKBR3 cells incubated first with free Trastuzumab (1 mg/ml) for 30 min
- incubated for 4 h with the different liposomal formulations
- no significant difference with the control liposomes in the presence or absence of free TRA (p-value = 0.095)
- for TRA-liposomes, calcein uptake was significantly hindered when HER2 receptors were first blocked with free TRA (p-value = 3.23×10^{-5})

➢ Result 8



- Flow cytometry analysis showing calcein fluorescence intensity inside SKBR3 cells incubated with the either the control or TRA-liposomes with or without prior incubation with free Trastuzumab (1 mg/ml)

Fluorescent microscopy imaging

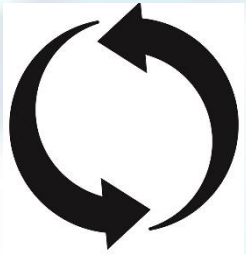
SKBR3 cells (2×10^5 cells/ml)
two 6-well plates

24 h

incubation with either
control or TRA-liposomes

4 h

One of the two plates was
sonicated (35 kHz) 5 min



The media was removed
were washed with PBS

fixed with 4% Formaldehyde

washing PBS buffer

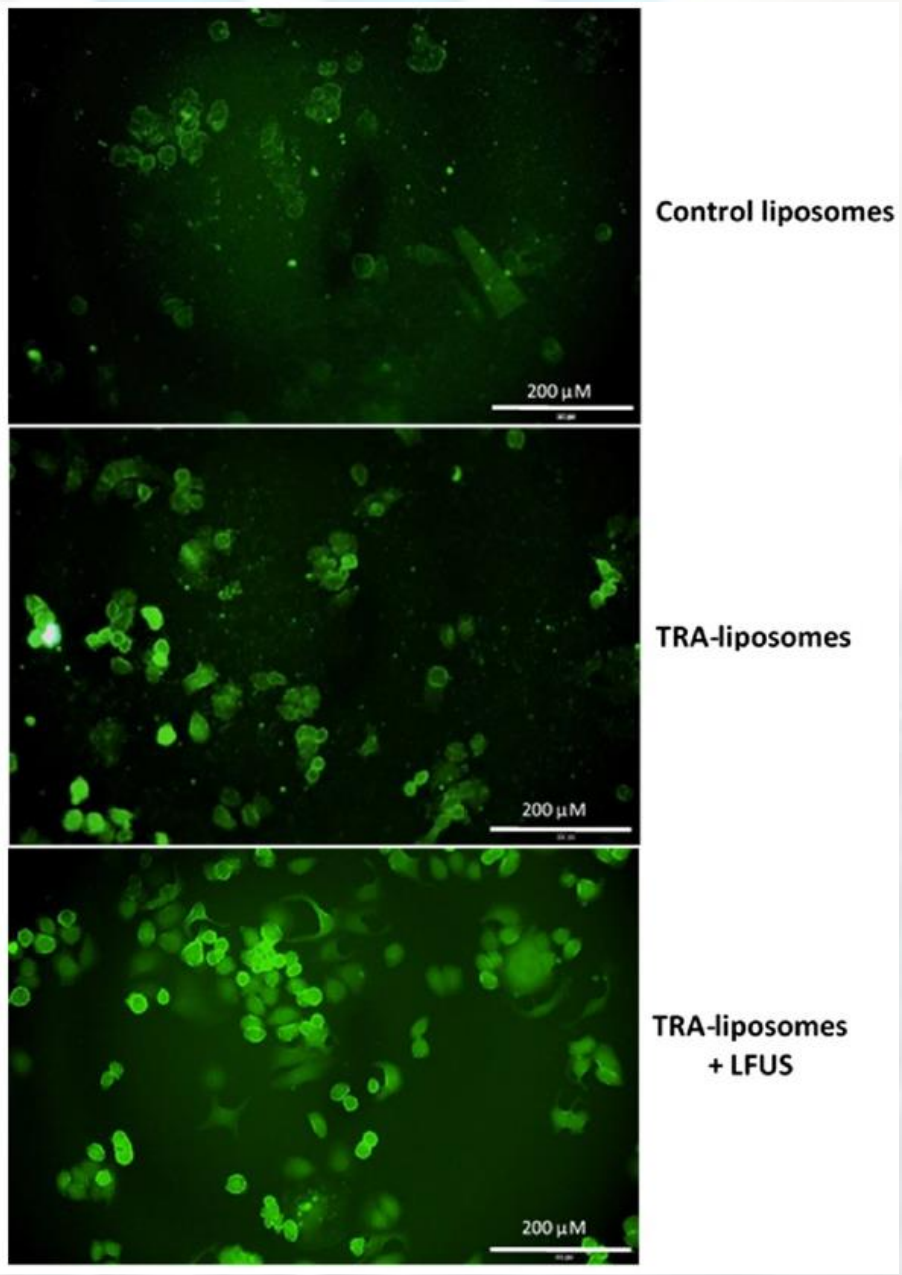
fluorescent microscope excitation filter at
470–495 nm and emissions at 510–550 nm

➢ Result 9

Fluorescent microscopic images

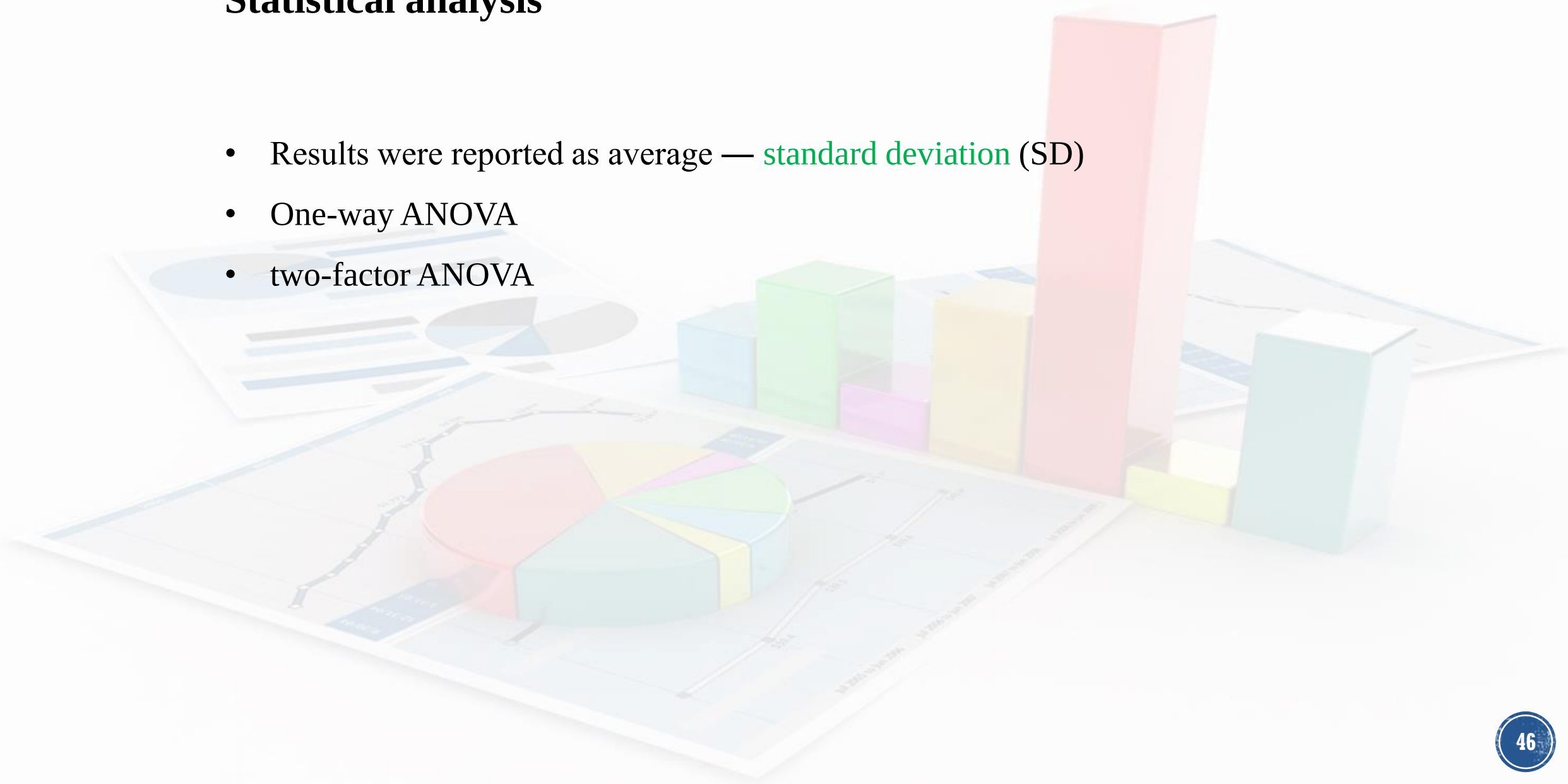
Fluorescence microscopy images of SKBR3 cells following 4 h incubation with

- control liposomes
- TRA liposomes encapsulating calcein
- TRA-liposomes exposed to LFUS (35-kHz) for 5 min

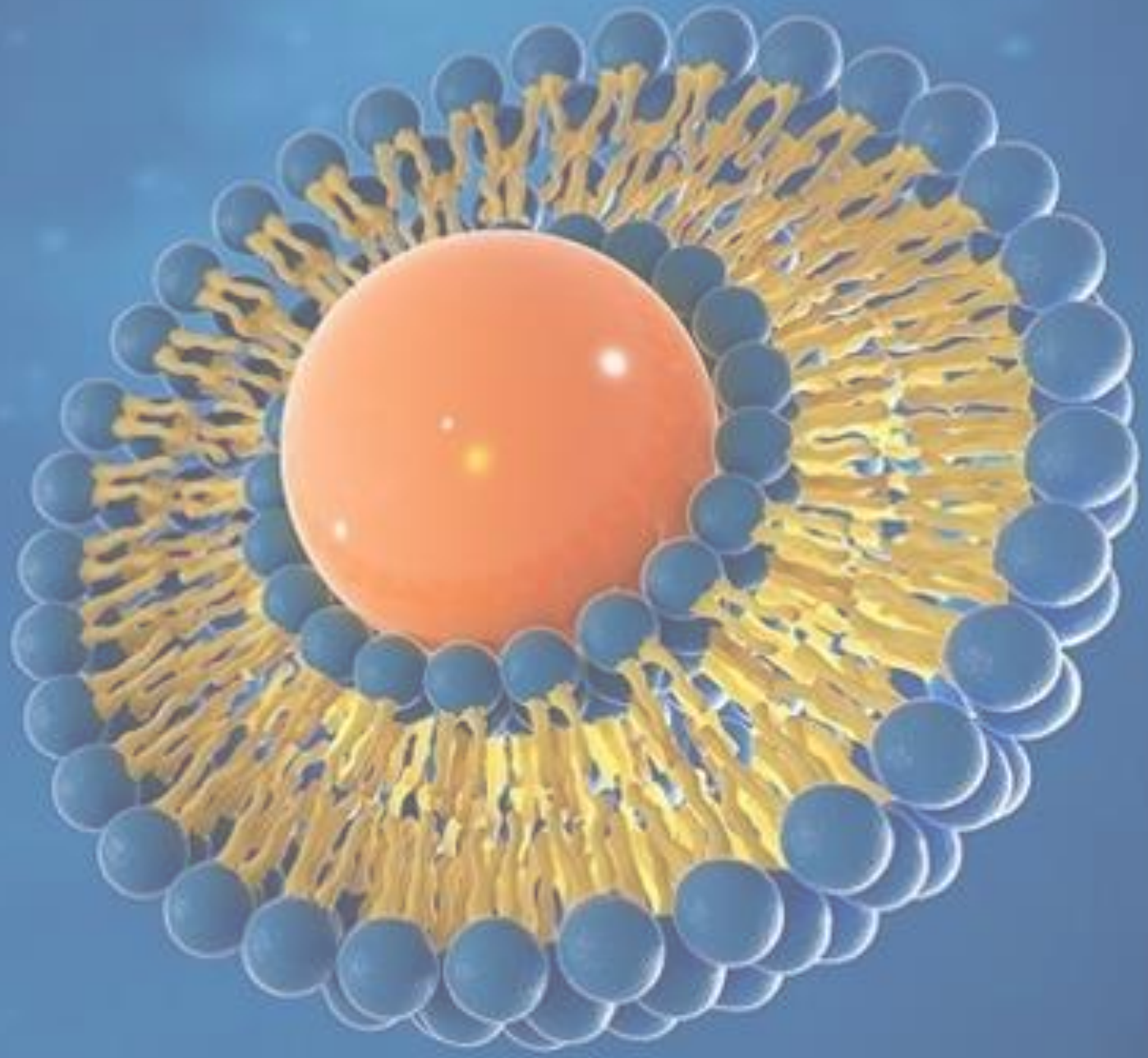


Statistical analysis

- Results were reported as average — standard deviation (SD)
- One-way ANOVA
- two-factor ANOVA



Discussion



- Targeted drug delivery is a promising technique in cancer treatment
- Liposomes are among the most successful nanocarriers ➡
  1. their high biodegradability
  2. biocompatibility
  3. ability to encapsulate both hydrophilic and hydrophobic drugs
- Antibodies are an attractive choice as targeting molecules conjugated to liposomes (immunoliposomes) to target certain receptors, such as HER2 receptors, overexpressed on the surface of breast cancer cells
- In this study ➡ conjugating a monoclonal antibody (trastuzumab) to pegylated liposomes and triggered with low-frequency ultrasound (LFUS) to enhance the release of the encapsulated drug

Immunoliposomes:

- enhance the efficiency
- reduce the off-target toxicity of the antineoplastic agent
- higher and more toxic concentration of one or more drugs to be delivered to the cells
- deliver large molecules such as genes

- Liposome-encapsulated doxorubicin was developed to reduce the toxicity while enhancing the targeting efficiency of DOX
- polyethylene glycol (PEG) → creates a hydrophilic shield that protects the liposomes from elimination and ensures a prolonged circulation time in the body
- Liposomal DOX has been studied widely over the past several years. Some liposomal formulations of DOX extensively evaluated for their safety and efficacy
- less cardiotoxicity and gastrointestinal toxicity
- Doxil (used in the US since 1995)
- Myocet (community authorization by the European Commission since 2000)

- Our synthesized immunoliposomes (TRA-liposomes) were within the recommended size (< 200 nm) to benefit from EPR effect
- LFUS (at 20 kHz) triggered calcein release from the liposomes
- the release increased with the increase in the US power density
- Previous studies ⇒ LFUS can trigger drug release from the liposomes in a controlled manner
- LFUS can produce both thermal and mechanical effects
- both may contribute to enhancing drug release from the liposomes

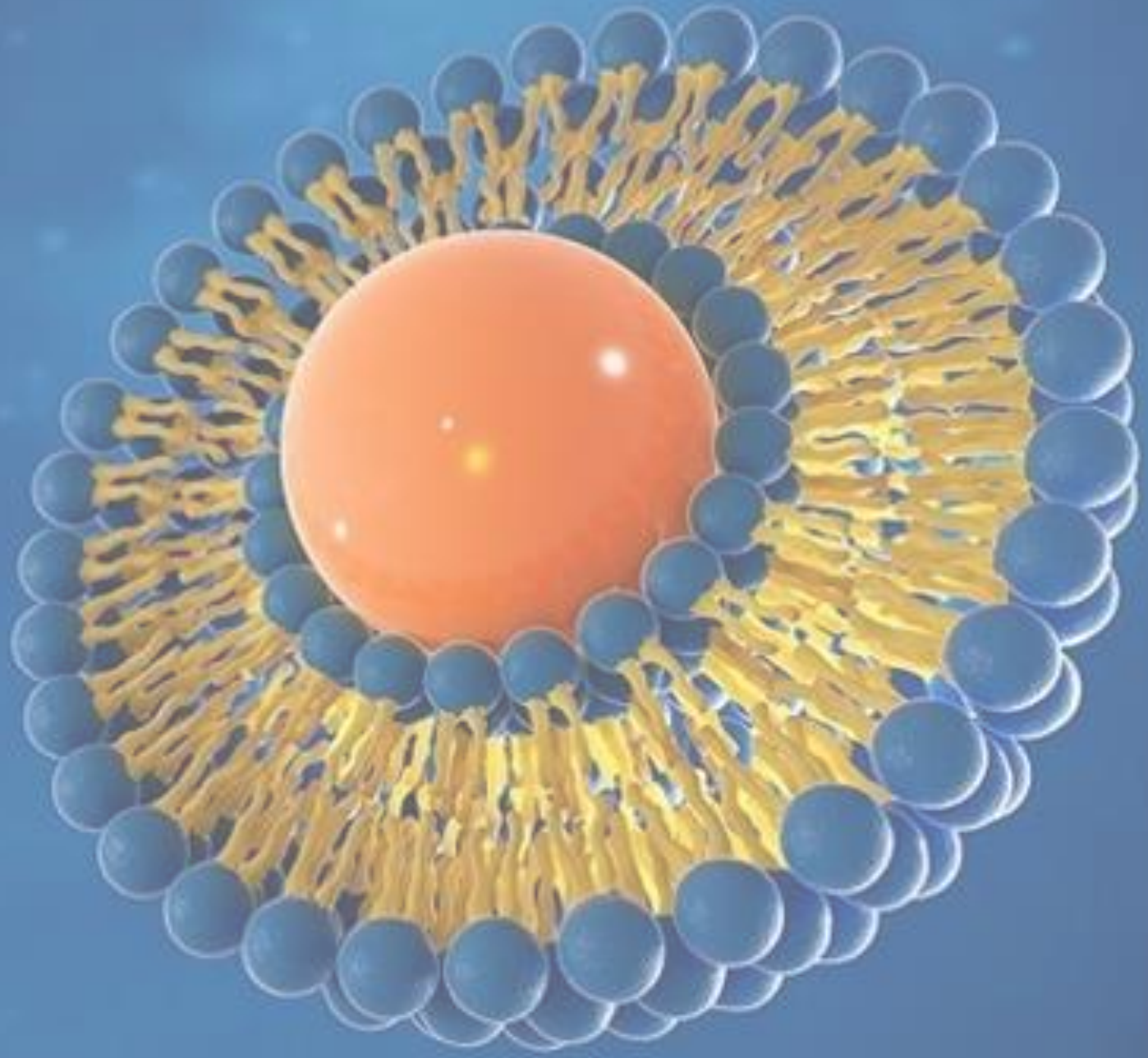
- When US was applied in our experiments, both types of liposomes released most of their content ≤ 31 °C
- lower than the transition temperature of DPPC (41.3 °C)
- the mechanical effects in the form of cavitation are the likely driving forces behind drug release
- according to the calculated MI values (below the threshold required for the collapse cavitation (MI = 0.3)) stable cavitation

- Cavitation may cause “sonoporation”
- which is the ability of US to modify cellular membranes’ permeability by creating transient pores in the membrane
- previous studies ⇒ cavitation-induced drug release from sonicated liposomes occured through pore formation rather than the destruction of the whole membrane
- LFUS application was not toxic to the cells
- confirms that sonoporation has no adverse effect on cell viability for the power density and frequency used in this study

- when the control liposomes were incubated with SKBR3 cells liposomes can transfer their load to the cytoplasm by fusing with the cellular membrane
- Vogel et al → this process is slow and increases gradually with time
- TRA-liposomes deliver their cargo to cells
- by membrane fusion
- by receptor-mediated endocytosis
- allow the cellular uptake of a larger number of liposomes in a shorter

- LFUS exposure has a visible effect on the action and uptake of free DOX due to the sonoporation effect
- the combination of immunoliposomes and US ⇒ a promising targeted technique to treat HER2 + primary or secondary breast cancer cell lines
- This can be achieved by:
- injecting the patients with immunoliposomes encapsulating the drug
- a clinically approved ultrasonic probe focuses acoustic waves on the tumor
- releasing the therapeutic agent
- causing the formation of pores in the cell membrane of the diseased cells

Conclusion



- The overexpression of HER2 receptors on the surface of some breast cancer cells provides a unique platform for HER2-targeted liposomes
- in this study → pegylated liposomes + monoclonal antibody Trastuzumab (TRA-liposomes) was synthesized
- the effect of applying LFUS to stimulate drug release
- in vitro results showed this method is a safe and effective technique in breast cancer treatment
- future in vivo and clinical work → unlock the full potentials of this promising therapeutic technique


Thank You
For Your Attention


Q & A